DRAFT: August 31, 1994

DECISION DOCUMENT
TSCA SECTION 5(H)(4) EXEMPTION FOR
CLOSTRIDIUM ACETOBUTYLICUM

I. SUMMARY

Clostridium acetobutylicum is an anaerobic, saccharolytic and proteolytic bacterium that has been isolated from a number of environments. The bacterium produces endospores which allows for long-term survival in the environment even in the presence of oxygen. It exists in the biologically inactive spore stage in soils except when vegetative growth is stimulated by anaerobiosis and other favorable growth conditions. Although other members of the genus produce some of the most lethal neurotoxins known, C. acetobutylicum is considered a benign microorganism. Throughout its long history of use for production of butanol and acetone, there have been no reports of adverse effects to human health or the environment. It is not pathogenic or toxigenic to humans, animals, or plants. The potential risks associated with the use of this bacterium in fermentation facilities are low.

II. BACKGROUND

A. Introduction

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA is proposing expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that would be exempt meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures for minimizing numbers of organisms emitted from the facility. When

balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

B. Criteria for Minimizing Release from Manufacturing Facilities

The standards prescribed for the Tier I exemption require the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is proposing, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's proposed standards for minimizing emissions specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the bacteria used in fermentation processes are usually debilitated,

either intentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment. Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is proposing that the number of microorganisms in fermentor gases be reduced by at least two logs prior to the gases being exhausted from the fermentor. EPA selected this number based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted in a gas from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also proposing that the requirements specify that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to (e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene procedures specified by EPA should protect most

individuals from the allergenic responses associated with microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement that entry be limited to essential personnel also addresses this consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for microorganisms qualifying for Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

C. Introduced Genetic Material Criteria

In order to qualify for either Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structural gene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for metabolizing multigenic toxins would be inadvertently introduced into a recipient microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the

complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform the microorganisms proposed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred and mobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should not prevent most microorganisms from meeting the exemption criteria, since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8}. Higher frequencies are likely only if the introduced genetic material has been altered or selected to enhance frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer

usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with Clostridium acetobutylicum, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of C. acetobutylicum will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of C. acetobutylicum, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of C. acetobutylicum, and EPA's review of the conditions selected.

D. Recipient Microorganism Criteria

Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place the microorganism on the list. The Agency's specific determination for Clostridium acetobutylicum is discussed in the next unit.

III. EVALUATION OF CLOSTRIDIUM ACETOBUTYLICUM

A. History of Use

1. History of safe commercial use. C. acetobutylicum has a long history of safe use in the industrial production of acetone and butanol in fermentation systems using maize mash, molasses, or other feedstocks. In the 1950's and 1960's, the lower costs associated with chemically-produced solvents and shortages of feedstocks led to the closing of microbial production plants in the U.S. and other countries. However, in recent years, production of acetone and butanol by C. acetobutylicum using agricultural and domestic wastes such as whey, wood shavings, bagasse, and rice straw has also been investigated. C. acetobutylicum is considered a Class 1 Containment agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules.

2. Products subject to TSCA. EPA has not yet received a submission for use of a strain of C. acetobutylicum under TSCA. However, the solvents produced by C. acetobutylicum, predominantly acetic acid, butyric acid, acetone, and butanol, are industrial chemicals that may have uses subject to TSCA. The bacterium also produces small amounts of lactic acid, ethanol, and succinate that could have uses subject to TSCA.

B. Identification of the Microorganism

1. Classification. The genus Clostridium consists of a large number of species with a wide range of biochemical and physiological traits. It has been suggested that the current genus, which is not well-defined, be subdivided into six groups because of its diversity. Although the number of simple phenotypic biochemical tests required to differentiate C. acetobutylicum from other species is quite large and not easily performed, there are several basic reactions which will separate C. acetobutylicum from other species. Within the species of C. acetobutylicum, the taxonomy is somewhat confusing. The five most commonly used strains differ widely with respect to their growth and their physiological, biochemical, and fermentative characteristics.

2. Related species of concern. Toxigenic members of the genus Clostridium produce some of the most lethal neurotoxins known, tetanus and botulinum toxins. C. botulinum is the causal agent of both food-borne and infant botulism. However, recently there have been reports of infant botulism caused by botulinum toxins produced by nontoxigenic clostridia, such as C. baratii and C. butyricum.

C. Risk Summary

1. Studies regarding potential for adverse effects. There are reports indicating that C. acetobutylicum is intermittently part of the normal flora of the human colon, although it does not appear to be a major component. There are no reports suggesting the C. acetobutylicum has the ability to produce mammalian toxins or enzymes known to be associated with virulence. The major threat to human health would be the acquisition of genes for toxins such as botulinum from toxigenic clostridia. Although there is no evidence in the literature that C. acetobutylicum can acquire genetic material from other bacteria, few studies on gene transfer have been conducted with this microorganism. It is theoretically possible, therefore, that like other nontoxigenic clostridia C. acetobutylicum could acquire the ability to produce toxins from toxigenic clostridia.

There are no reports that C. acetobutylicum is an animal or a plant pathogen. However, C. acetobutylicum produces a bacteriocin near the end of the exponential growth stage. This bacteriocin is inhibitory to other C. acetobutylicum strains as well as C. felsineum. Although C. acetobutylicum fixes atmospheric nitrogen, the amount of nitrogen fixed by this organism in the environment would probably be negligible, as it is expected to be present predominantly as spores.

2. Studies regarding survival in the environment. No studies exist on the survival of C. acetobutylicum specifically, although it has been isolated from many environments. C. acetobutylicum is an obligate anaerobe and growth is inhibited in the presence of oxygen. Typically C. acetobutylicum exists predominantly as endospores which are quite resistant to adverse environmental conditions and can survive for many years.

IV. BENEFITS SUMMARY

Substantial benefits are associated with this proposed exemption. Clostridium acetobutylicum, is already widely employed in general commercial uses, some of which are subject to TSCA reporting. The Agency believes this exemption will result in resource savings both to EPA and industry without compromising the level of risk management afforded by the full 90 day review. In addition to assessing the risk of C. acetobutylicum, EPA has developed criteria limiting the potential for transfer of and expression of toxin sequences, and the conditions of use specified in the exemption are met (Tier I) or will be reviewed by EPA to ensure adequate risk reduction (Tier II). EPA requirements for minimizing numbers of viable microorganisms

emitted are within standard operating procedures for the industry, and both the procedures and the structures specified in the exemption are the type industry uses to protect their products from contamination.

The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

V. RECOMMENDATION AND RATIONALE

A. Recommendation

Clostridium acetobutylicum is recommended for a TSCA section 5(h)(4) tiered exemption.

B. Rationale

1. Risks from use of the recipient microorganism Clostridium acetobutylicum are low. C. acetobutylicum is a benign microorganism. Industrial strains have a long history of safe use in the production of acetone and butanol with no incidents of adverse effects to human health or the environment. There is a theoretical possibility that C. acetobutylicum could acquire toxin genes from one of the toxigenic clostridia. However, the likelihood of acquisition of a toxin gene by an industrial strain of C. acetobutylicum with a history of safe use in an industrial setting is remote, because care is taken to prevent contamination of the fermentors by other microorganisms. Under good industrial practices, fermentation workers would be expected to be wearing protective clothing and participating in good industrial hygiene which would reduce the risk of exposure to toxins potentially produced by C.acetobutylicum. Releases of this microorganism would not pose any significant ecological hazards, because this organism is already widespread in the environment and it is not pathogenic to animals or plants.

2. Use of strains of Clostridium acetobutylicum which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. C. acetobutylicum presents low risk of adverse effects to human health or the environment. Because the recipient microorganism was found to have little potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase

potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption. When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

REQUEST FOR COMMENTS

The Risk Assessment to support the proposal of Clostridium acetobutylicum as a candidate for the TSCA section 5(h)(4) tiered exemption recommends that only asporogenic strains with a sporulation deficiency of at least 10^{-7} be eligible for the exemption. This Decision Document recommends all strains of C. acetobutylicum for this exemption. The recipient microorganism C. acetobutylicum was found to have little potential for adverse effects. The probability is low that the insertion of genetic material meeting EPA's criteria into such a microorganism will change its behavior so that it would acquire the potential for causing adverse effects. Therefore, there should be no need to restrict this exemption to asporogenic strains.

However, because there is a discrepancy in the recommendations of the Risk Assessment and the Decision Document, EPA requests comment on whether its current recommendation of all strains of C. acetobutylicum as eligible for this exemption should be modified to limit the exemption only to asporogenic strains.

Additionally, no data were available for assessing the releases specifically from fermentation facilities using C. acetobutylicum, because commercial anaerobic fermentation processes are currently at the research stage of development. The assessors indicated that releases from an anaerobic process are expected to be no higher than those for an aerobic system. Therefore, for purposes of the Risk Assessment for C. acetobutylicum, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated based on the same information used for the aerobic microorganisms proposed for the TSCA section 5(h)(4) exemption. EPA requests comment on whether the assumption that releases from an anaerobic process would not be higher than those for an aerobic process is an appropriate assumption for the purposes of this assessment. Anyone possessing information on assessing releases from anaerobic processes is encouraged to submit such information to EPA.

Attachment 1:

INTEGRATED RISK ASSESSMENT OF

CLOSTRIDIUM ACETOBUTYLICUM

I. INTRODUCTION

Clostridium acetobutylicum is an anaerobic, saccharolytic and proteolytic bacterium that has been isolated from a number of environments. The bacterium produces endospores which allows for long-term survival in the environment even in the presence of oxygen. It exists in the biologically inactive spore stage in soils except when vegetative growth is stimulated by anaerobiosis and other favorable growth conditions. Although other members of the genus produce some of the most lethal neurotoxins known, *C. acetobutylicum* is considered a benign microorganism. Throughout its long history of use for production of acetone and butanol, there have been no reports of adverse effects to human health or the environment. It is not pathogenic or toxigenic to humans, animals, or plants. The potential risks associated with the use of this bacterium in fermentation facilities are low.

History of Commercial Use and Products Subject to TSCA Jurisdiction

C. acetobutylicum has a long history of safe use in the industrial production of acetone and butanol in fermentation systems using maize mash, molasses, or other feedstocks. Jones and Woods (1986) have thoroughly documented its history of use for solvent production. Between 1912 and 1914, Weizmann isolated a number of cultures capable of producing acetone and butanol, the most efficient of which was designated BY and later named *C. acetobutylicum*. With the outbreak of World War I and the need for acetone in munitions manufacturing, a plant was erected in England in 1916, and several existing distilleries were recruited for acetone production using maize as a substrate. However, the German blockade affected the supply of grain which made it necessary to erect another plant where grain was more readily available. An existing distillery in Canada was recruited for acetone production using the Weizmann process and strain. This plant remained in operation until the end of the war. When the United States entered the war in 1917, several plants were established in Indiana. These plants were also closed at the end of the war when the need for acetone diminished. However, the rapidly expanding automobile industry after the war created a market for butanol as a solvent in nitrocellulose lacquers for

car finishes. The plants in Indiana were reopened and additional plants were built. In 1936, the patent on the Weizmann strain expired, and new plants were built in Baltimore, MD, Philadelphia, PA, and in Puerto Rico. After 1936, acetone-butanol fermentation plants were also constructed in Japan, India, Australia, and South Africa. It is unknown whether these additional plants utilized *C. acetobutylicum* or other solventogenic clostridia. In the 1950's and 1960's, the lower costs associated with chemically-produced solvents and shortages of feedstocks led to the closing of microbial production plants in the United States and in other countries. The plant in South Africa which presumably utilized a strain of *C. acetobutylicum*, P262, remained in operation until the early 1980's (Jones and Woods, 1986). It is unknown whether plants in other countries are still in production. In recent years, production of acetone and butanol by *C. acetobutylicum* in the utilization of agricultural and domestic wastes such as whey, wood shavings, bagasse, and rice straw has also been investigated (McNeil and Kristiansen, 1986).

The solvents produced by *C. acetobutylicum*, predominately acetic acid, butyric acid, acetone, and butanol are industrial chemicals that may be subject to TSCA. The bacterium also produces small amounts of lactic acid, ethanol, and succinate that could also be subject to TSCA.

II. IDENTIFICATION AND TAXONOMY

A. Overview

C. acetobutylicum is a saccharolytic and proteolytic bacterium that has been isolated from soils, lake sediments, well water, clam gut, and from bovine, canine, and human feces (Cato et al., 1986). Although gram positive at early stages of growth, it is pleomorphic at later stages. This bacterium is rod-shaped, motile by peritrichous flagella, and produces subterminal endospores. *C. acetobutylicum* is an obligate anaerobe, and therefore, will not grow in the presence of oxygen. However, vegetative cells may survive oxygen exposure for several hours (Gottschalk et al., 1981). The resistant endospores produced by this bacterium enable it to survive in the environment for many years, even in the presence of oxygen. *C. acetobutylicum* is capable of fixing atmospheric nitrogen, and some strains produce inducible carboxymethyl cellulase and cellobiase enzymes (Cato et al., 1986).

B. Taxonomy and Characterization

The genus *Clostridium*, which was first described in 1880, consists of a large number of species with a wide range in biochemical and physiological traits (Cato et al., 1986). There are only four criteria that need to be met for an isolate to be assigned to the genus *Clostridium*. These are: (1) the ability to form endospores, (2) anaerobic energy metabolism, (3) the inability for dissimilatory sulfate reduction, and (4) possession of a gram positive cell wall (that may react gram negative) (Andreesen et al., 1989). It has been suggested that the current genus, which is not well-defined, be subdivided into six groups because of its diversity (Cato and Stackebrandt, 1989). According to Gottschalk et al. (1981), there is no standard classification system for the genus *Clostridium*, and in many cases with the nonpathogenic species, the taxonomic descriptions are incomplete and are based on single strains only. Historically, species in the genus were defined more on functional grounds, i.e., their role in the environment or ability to cause human infection, rather than on a solid phenotypic or genotypic basis. In recent years, the species in the genus have been better delineated. *C. acetobutylicum* is listed in Bergey's Manual of Systematic Bacteriology as a distinct species (Cato et al., 1986). Although the number of simple phenotypic biochemical tests required to differentiate this species from others is quite large and not easily performed, there are several basic reactions which will separate *C. acetobutylicum* from the human pathogenic species. The group of histolytic clostridia, including *C. perfringens* and *C. histolyticum*, and the major toxin producers, *C. botulinum* and *C. tetani*, all produce extracellular proteinase with gelatin as a substrate while *C. acetobutylicum* does not. Therefore, there is little chance of confusing *C. acetobutylicum* with the most pathogenic species of the genus (Edberg, 1991).

In regards to other nonpathogenic species, *C. acetobutylicum* supposedly can be differentiated from *C. baratii*, as the latter produces large amounts of lactic acid in addition to acetic and butyric acids. However, *C. acetobutylicum* does produce some lactic acid. Likewise, *C. butyricum* produces large amounts of formic acid in addition to acetic and butyric acids, whereas *C. acetobutylicum* does not produce formic acid (Edberg, 1991). However, McNeil and Kristiansen (1986) stated that the taxonomy of solventogenic clostridia based on end product analysis is an unreliable means of species classification because solvent production is a highly variable trait (George et al., 1983), and solvent production can change or be manipulated depending on the composition of the fermentation medium.

According to Johnston and Goldfine (1983), related clostridial species could be separated by thin layer

chromatography based on their principal phospholipids and the degree of saturation of those lipids. It appears, therefore, that it may be possible to differentiate closely related species based on fatty acid and lipid analysis. However, these methods are not routinely available nor have these methods been applied to industrial strains of *C. acetobutylicum* (Edberg, 1991).

Even within the species of *C. acetobutylicum*, the taxonomy is somewhat confused. The five most commonly used strains of *C. acetobutylicum* strains differ widely with respect to their growth and their physiological, biochemical, and fermentative characteristics (Woolley and Morris, 1990). It has been suggested that there are at least two groups within the species (Woolley and Morris, 1990). There is apparently little DNA homology between the two proposed groups, the first of which would consist of strains NCIB 8052 and P262 (a widely used industrial strain) and the other group which would contain the type strain, ATCC 824, and DSM 1731 (Woolley, 1988). Note that in the 1989 ATCC Catalogue of Bacteria and Phages (Gherna, 1989) and in an earlier publication (Gottschal and Morris, 1982), ATCC 824 and NCIB 8052 were stated as being identical strains, but in the above proposed classification, they would fall into different DNA homology groups. Strain N1-4, formerly known as *C. saccharoperbutylacetonicum* but presently designated as *C. acetobutylicum*, apparently does not fit well into either group. Not all strains of *C. acetobutylicum* have been compared for DNA homology, nor have phylogenic studies using RRNA sequencing been done.

C. Related Species of Concern

The taxonomic uncertainty associated with *C. acetobutylicum* is of concern in that closely related nontoxigenic clostridia have acquired the ability to produce toxins. Toxigenic members of the genus produce some of the most lethal neurotoxins known, tetanus and botulinum toxins (Shone and Hambleton, 1989). Typically, *C. botulinum* is the causal agent of both food-borne and infant botulism. In food-borne botulism, the pre-existing toxin is ingested and absorbed. In infant botulism, there is colonization and multiplication of the organism in the intestinal tract of the infant, where the toxin is then produced and absorbed (Hall et al., 1985). Recently, there have appeared three reports of infant botulism caused by botulinum toxin types E and F, that were produced not by *C. botulinum* but by nontoxigenic clostridia. In these three cases, typically nonpathogenic, nontoxigenic organisms identified as *C. baratii* and *C. butyricum* were shown to be capable of producing the botulism type F and type E toxins, respectively, (Hall et al., 1985; McCroskey et al., 1986; Aureli et al., 1986). However, as

previously stated, it is possible to taxonomically distinguish *C. acetobutylicum* not only from the toxigenic species such as *C. botulinum* and *C. tetani*, but also from other closely related solventogenic species such as *C. butyricum* and *C. baratii*.

III. HAZARD ASSESSMENT

A. Human Health Hazards

There are several reports in the literature that suggest that *C. acetobutylicum* is, at least intermittently, part of the normal flora of the human colon, although it does not appear to be a major component (McNeil and Kristiansen, 1986; Jones and Woods, 1986; Awang et al., 1988). Except for its isolation from human feces, *C. acetobutylicum* has not been associated with humans. There are no reports in the literature suggesting that *C. acetobutylicum* has the ability to produce mammalian toxins, nor does it produce enzymes known to be associated with virulence. It apparently does not produce any extracellular or intracellular materials that would be toxic to humans. A search of a list of toxic substances produced by bacteria failed to reveal this species (Gill, 1986). Since *C. acetobutylicum* apparently produces no virulence factors, one would expect an extraordinarily large number of microorganisms to be required to cause even a superficial infection (Edberg, 1991).

The major threat to human health would be the acquisition of toxin production genes from botulism toxin-producing members of the genus. As mentioned previously, closely related species, *C. baratii* and *C. butyricum*, have been shown to acquire the botulism toxin production genes from clostridial pathogens. An isolate of *C. baratii* was repeatedly isolated from the stool of an infant suffering from infant botulism (Hall et al., 1985). McCroskey et al. (1986) and Aureli et al. (1986) described the isolation of *C. butyricum* from infants with type E botulism. The Center for Disease Control further analyzed these strains of *C. baratii* and *C. butyricum* and confirmed that they did, in fact, produce type F and type E neurotoxins that were similar to, or indistinguishable from, those produced by *C. botulinum*. Gimenez and Sugiyama (1988) studied the type E toxin produced by *C. butyricum* and found it was very similar to that produced by *C. botulinum*, and that the LD_{50} for mice and the immunological cross-reactivity were extremely similar. The identification of both these organisms in question was confirmed by DNA reassociation studies with the type strains from these species (Suen et al., 1988). Therefore, it appears that closely related clostridial species can acquire the ability to produce botulinum toxins. Presumably, these toxins are coded for by extrachromosomal elements such as

plasmids. Although there is no evidence in the literature that *C. acetobutylicum* can acquire genetic material from other bacteria, few studies on gene transfer have been conducted with this microorganism. It is theoretically possible, therefore, that *C. acetobutylicum*, like *C. baratii* and *C. butyricum*, could acquire the ability to produce toxins from toxigenic clostridia.

Infant botulism, however, is a rare disease. The Center for Disease Control estimates that there are approximately 100 confirmed cases of infant botulism per year. It seems remote that the events necessary for an infant to acquire botulism from *C. acetobutylicum* would occur in an industrial setting. First, the strain would have to acquire the genetic basis to elaborate a botulinum toxin. Second, an infant present in the fermentation facility would have to ingest a large number of spores with the ability of the spores to form viable vegetative cells in the immature pediatric gastrointestinal system. Good industrial practices would certainly preclude the presence of infants in fermentation facilities. Consequently, the concern for infant botulism from the industrial use of this species is remote (Edberg, 1991).

There is, however, also a condition known as wound botulism following trauma, whereby a large number of spores are inoculated into an open wound. Typically, this disease is caused by *Clostridium botulinum*. If *C. acetobutylicum* strains acquired the ability to produce botulism toxin as did the other nonpathogenic species mentioned above, there is a possibility that high concentrations of vegetative cells or spores could accidentally be inoculated into a wound. However, good worker hygiene, including use of protective clothing, should mitigate this concern.

The likelihood of toxin acquisition by an industrial strain with a history of safe use in an industrial setting seems remote. The only concern is that new strains or environmental isolates of *C. acetobutylicum* may have had contact with clostridial pathogens and acquired toxin-production genes. It may be prudent for the manufacturer to screen culture supernatants at late log phase of growth for the production of botulism toxins (Edberg, 1991). Since it is highly unlikely that *C. acetobutylicum* would acquire botulinum toxin genes, the overall human health risk of *C. acetobutylicum* is minimal (Edberg, 1991).

B. Environmental Hazards

1. Hazards to Animals

There are no reports in the literature suggesting that *C. acetobutylicum* is an animal pathogen (McClung, 1991), and it is not listed as such in a review of animal pathogens by Hill (1981). As mentioned above, *C. acetobutylicum* has not been shown to produce any toxins, enzymes, or virulence factors typically associated with mammalian toxicity or pathogenicity (Edberg, 1991).

The only remote hazard to animals would be the acquisition of botulism toxin genes as shown for closely related solventogenic clostridia. Botulism toxins have been shown to be toxic to mice (Cato et al., 1986), and the supernatant culture fluid from strains of type E botulism toxin-producing *C. botulinum* were toxic to gallinaceous birds (pheasants, turkeys, grouse, and domestic fowl) (Gross and Smith, 1971).

2. Hazards to Plants

There are no reports in the literature indicating that *C. acetobutylicum* has any adverse effects on plants. No members of the genus *Clostridium* are listed as plant pathogens according to the Federal Plant Pest Act (7 CFR 330, et seq.)

3. Hazards to Other Microorganisms

C. acetobutylicum (P262) produces a bacteriocin (Barber et al., 1979) near the end of the exponential growth stage. Bacteriocins are usually thought to have bactericidal action against the same species or other clostridial species. Barber et al. (1979) reported that this bacteriocin had inhibitory effects against members of the same species and against one other clostridial species, *C. felsineum*. The bacteriocin from *C. acetobutylicum* (P262) was not inhibitory to *Achromobacter*, *Escherichia coli, Serratia marcescens*, *Salmonella typhimurium*, or *Bacteroides fragilis* (Barber et al., 1979). Soucaille and Goma (1986) reported that a bacteriocin obtained from *C. acetobutylicum* strain ATCC 824 was similar to that obtained from strain P262 (Barber et al., 1979). The bacteriocin from ATCC 824 was not inhibitory to *Corynebacterium glutamicum, E. coli, Proteus mirabilis, Aerobacter aerogenes*, or *Zymomonas mobilis*, but was inhibitory to *C. butyricum* and members of the family Bacillaceae including *Bacillus subtilis* and *B. megaterium* (Soucaille and Goma, 1986). The production of bacteriocins in the environment by *C. acetobutylicum* would not be likely to be of concern. Although *C. acetobutylicum* is expected to survive in the environment, it will exist predominantly as spores rather than as vegetative cells since it is obligately anaerobic. Even if bacteriocins are released into the environment with spent

fermentation wastes, there is still little environmental concern due to the high numbers of bacilli typically found in soils. The levels of members of the genus *Bacillus* are thought to range between 10^6 - 10^7 per gram of soil (Alexander, 1977). In addition, *C. acetobutylicum* is widespread in the environment. Under some conditions, population levels of 10^6 clostridia per gram of soil have been found (Alexander, 1977).

4. Hazards Posed to Other Processes

Although *C. acetobutylicum* fixes atmospheric nitrogen, the amount of nitrogen fixed by this organism in the environment would probably be negligible, as it is expected to survive predominately as spores. In addition, the amount of nitrogen fixed by nonsymbiotic microorganisms is relatively small compared to symbiotic associations. The numbers of N_2-fixing clostridia in arable soils range from 10^2 to 10^6 per gram of soil, of which *C. acetobutylicum* is thought to be one of three prominent species (Alexander, 1977). However, in the environment, nitrogen fixation rates would not be expected to be appreciable, as the efficiency of nonsymbiotic nitrogen fixation is low and energy sources are scarce.

Some strains of *C. acetobutylicum* produce cellulases which enable the fermentation of some feedstocks, but this does not appear to be a potential environmental hazard since this organism already exists in the environment (Alexander, 1977), and survival would most likely be in the spore stage.

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

C. acetobutylicum is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986).

According to Reilly (1991) no data were available for assessing the releases specifically from fermentation facilities using *C. acetobutylicum* because commercial anaerobic fermentation processes are currently at the research stage of development. The releases from an anaerobic process are expected to be no higher than those for an aerobic system (Reilly, 1991; Macek, 1992). Therefore, for purposes of this assessment, the potential worker exposures and routine releases to the environment from large-scale, conventional aerobic fermentation processes estimated on information available from eight premanufacture

notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms will be used (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the processes were considered typical of fermentation process technology. Area samples were taken in locations where the potential for worker exposure was considered to be potentially greatest, ie. near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use area monitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Fate of the Organism

No studies exist in the literature on the survival of *C. acetobutylicum* specifically, although it has been isolated from many environments (Cato et al., 1986). This bacterium is an obligate anaerobe and growth is inhibited in the presence of oxygen. However, vegetative cells can survive exposure to oxygen for several hours (Gottschalk et al., 1981). Typically, *C. acetobutylicum* exists as endospores which are quite resistant to

adverse environmental conditions and can survive for many years. Although no specific survival studies exist on this bacterium, its widespread presence in nature, its ability to colonize anaerobic environments, and the resistance of its endospores indicate that released microorganisms are likely to survive outside of containment (Versar Inc., 1992).

2. Releases

Estimates of the number of viable *C. acetobutylicum* organisms released per production batch are presented in Table 1. All calculations are based on the use of asporogenic strains, with a sporulation deficiency of 10^{-7}. The minimally controlled scenario assumes no treatment of fermentor off-gas and assumes a 2 log reduction relative to the maximum cell density of the fermentation broth resulting from inactivation (Reilly, 1991). The full exemption scenario assumes the use of in-line filters to treat vent gases and a 99% removal efficiency under normal operating conditions. It also assumes an overall 6 log reduction relative to the maximum cell density of the fermentation broth resulting from inactivation steps (Reilly, 1991).

TABLE 1. Estimated Number of Viable Clostridium acetobutylicum Organisms Per Production Batch

Release Media	Minimally Controlled (cfu/day)	Full Exemption (cfu/day)	Release (days/year)
Air Vents	$2x10^{8}$ - $1x10^{11}$	$2x10^{6}$ - $1x10^{9}$	350
Rotary Drum Filter	250	250	350
Surface Water	$7x10^{13}$	$7x10^{9}$	90
Soil/Landfill	$7x10^{15}$	$7x10^{11}$	90

Source: Reilly, 1991

In addition to the releases estimated in Table 1, spores would be released at a rate of 1.7 X 10^{10} spores/day in solid wastes and 2 X 10^{8} spores/day in aqueous wastes assuming that the strains are asporogenic with a sporulation deficiency of 10^{-7} (Versar Inc., 1992). In addition, these estimates are worst-case estimates that assume that the inactivation procedure is not effective for those spores produced by sporulation deficient strains and the separation efficiency for the rotary drum filter is 99 percent (Reilly, 1991).

3. Air

There are no specific data which indicate the survivability of *C. acetobutylicum* in the atmosphere after release. Although this organism exhibits an intolerance towards oxygen and would not be expected to proliferate under aerobic conditions, it has been found to survive oxygen exposure for several hours (Gottschalk et al., 1981). This and its ability to produce spores suggests that survival may occur outside of containment (Versar Inc., 1992).

Releases to air from fermentor off-gas could potentially result in nonoccupational inhalation exposures due to point source releases. To estimate exposures from this source, the sector averaging form of the Gaussian algorithm described in Turner (1970) was used. For the purposes of this assessment, a release height of 3 meters and downward contact at a distance of 100 meters were assumed. Assuming that there is no removal of organisms by additional treatment of off-gases, potential human inhalation dose rates are estimated to range from 3.0×10^3 to 1.5×10^6 cfu/year for minimally controlled systems and 3.0×10^1 to 1.5×10^4 cfu/year for systems with full exemptions. It should be noted that these estimates represent hypothetical exposures under reasonable worst-case conditions.

4. Water

The concentrations of *C. acetobutylicum* in surface waters were estimated using stream flow values for water bodies receiving process wastewater discharges from facilities within SIC code 283 (drugs, medicinal chemicals, pharmaceuticals). The surface water release data (cfu/day) shown in Table 1 were divided by the stream flow values to yield a surface water concentration of the organism (cfu/L). The stream flow values for SIC Code 283 were based on discharger location data retrieved from the Industrial Facilities Discharger (IFD) database on December 5, 1991, and surface water flow data retrieved from the RXGAGE database. Flow values were obtained for water bodies receiving wastewater discharges from 154 indirect (facilities that send their waste to a POTW) and direct dischargers (facilities that have a NPDES permit to discharge to surface water). Tenth percentile values indicate flows for smaller rivers within this distribution of 154 receiving water flows, and 50th percentile values indicate flows for more average rivers. The flow value expressed as 7Q10 is the lowest flow observed over seven consecutive days during a 10-yr. period. The use of this methodology to estimate concentrations of *C. acetobutylicum* in surface water assumes that all of the discharged organisms survive wastewater treatment and that growth is not enhanced by

any component of the treatment process. Table 2 gives the estimated concentrations of *C. acetobutylicum* in surface water for minimally controlled and full exemption scenarios (Versar, 1992).

TABLE 2. Clostridium acetobutylicum Concentrations in Surface Water

Flow	Receiving Stream Flow (MLD*)		Organisms (cfu/l)	
	Mean	Q710	Mean	Q710
Minimally Controlled				
10th Percentile	156	5.60	4.5×10^{5}	1.25×10^{7}
50th Percentile	768	68.13	9.11×10^{4}	1.03×10^{6}
Full Exemption				
10th Percentile	156	5.60	4.5×10^{1}	1.25×10^{3}
50th Percentile	768	68.13	9.11×10^{0}	1.03×10^{2}

*MLD = million liters per day
Source: Versar, 1992

The concentrations of *C. acetobutylicum* spores in surface water were also estimated using the methodology and assumptions described above. Estimated concentrations of spores in surface water are presented in Table 3.

TABLE 3. Concentrations of Clostridium acetobutylicum spores in surface water

Flow	Spores/l	
	Mean	7Q10
l0th Percentile	1.28×10^{0}	3.57×10^{1}
50th Percentile	2.60×10^{-1}	2.93×10^{0}

Source: Versar, 1992

5. Soil

Since soil is a natural habitat for *C. acetobutylicum*, long-term survival in soil may be expected to occur, particularly under anaerobic conditions. The resistant endospores formed

could promote survival for years, even under aerobic conditions. Human exposures via dermal and ingestion routes, and environmental exposures (i.e., to terrestrial, avian, and aquatic organisms via runoff) may occur at the discharge site because of the potential establishment of *C. acetobutylicum* within the soil.

V. INTEGRATION OF RISK

A. Discussion

C. acetobutylicum is a common soil proteolytic and saccharolytic bacterium which is widespread in nature. Population levels of clostridia, of which *C. acetobutylicum* is thought to predominate, range from 10^2 to 10^6 per gram of soil. This bacterium is obligately anaerobic implying growth only under reducing conditions, although the vegetative cells have been shown to survive for several hours with oxygen exposure. It exists predominately in the environment as endospores which are quite resistant to adverse environmental conditions such as heat, desiccation, low nutrient status, and aerobic conditions. Releases from the fermentation facility of vegetative cells into reducing environments, or of spores into any environment, would, most likely, result in survival of the bacterium.

There are no reports of ecological or human health hazards caused by *C. acetobutylicum*. This bacterium is not thought to be a pathogen of either plants or animals. It does not produce any toxins, enzymes, or virulence factors normally associated with mammalian toxicity. Although it can, intermittently, occupy the human intestines, it is not thought to be a major component of the normal human flora. Except for its isolation from human feces, it is not otherwise associated with humans.

The only potential risk associated with *C. acetobutylicum* is the possibility of acquiring toxin-producing genes from pathogenic clostridia. *Clostridium botulinum* and *C. tetani* produce some of the most lethal neurotoxins known. There are no reports in the literature indicating that *C. acetobutylicum* can acquire these toxin genes. However, other closely related solventogenic, nontoxigenic clostridia have acquired the ability to produce botulism toxin types E and F. There are three cases of infant botulism which were caused by a strain of *C. baratii* and two strains of *C. butyricum*. The botulism toxins produced by these typically nontoxigenic clostridia were indistinguishable from the botulism toxins produced by *C. botulinum*. The toxin genes in *C. botulinum* are presumably coded for by extrachromosomal elements such as plasmids. It appears that the nonpathogenic *Clostridium* species acquired the toxin-producing genes from *C. botulinum*. Although there are no reports indicating that *C. acetobutylicum* can acquire toxin genes, only a limited

number of gene transfer studies have been conducted with this bacterium. It is theoretically possible that *C. acetobutylicum* could acquire botulism toxin genes.

The only concern for the acquisition of botulism toxin genes lies with the use of strains or environmental isolates that may have been in contact with the toxigenic bacteria. The likelihood of acquisition of a toxin gene by an industrial strain of *C. acetobutylicum* with a history of safe use in an industrial setting is remote due to the fact that care is taken to prevent contamination of the fermentors by other microorganisms. The only cases in which the closely related nontoxigenic clostridia acquired the toxin genes involved infant botulism. The conditions needed for development of infant botulism would not occur in fermentation facilities as exposure to large numbers of spores directly by the infant is necessary. Wound botulism of industrial workers is also highly unlikely since large numbers of vegetative cells or spores need to be inoculated into an open wound. Under good industrial practices, the fermentation workers would be expected to be wearing protective clothing and participating in good industrial hygiene which would allay this risk of infection given an accidental spill. All of these scenarios for botulism toxicity are dependent on the acquisition of the toxin gene by the organism which is theoretically possible, but has never been shown.

Industrial strains of *C. acetobutylicum* have a long history of safe use in the production of acetone and butanol with no incidents of adverse effects to human health or the environment. This organism is considered benign, and the only associated potential hazard with it is the theoretical possibility of acquisition of botulism toxin genes from toxigenic clostridia. Toxin acquisition has never been reported for this particular species, and the likelihood is remote, especially in an industrial setting where efforts are taken to minimize contaminants of the fermentation. If released into the environment, this bacterium is expected to survive, predominately as resistant spores except under anaerobic conditions. However, naturally-occurring *C. acetobutylicum* strains are widespread in the environment, and the limited exposure resulting from the use of *C. acetobutylicum* in fermentation facilities will not affect the population size of this species in the environment. Both the hazard and the exposure associated with the use of *C. acetobutylicum* are low. Therefore, the risks to human health and the environment associated with the use of this microorganism are low.

B. Recommendations

Asporogenic strains, with a sporulation deficiency of 10^{-7}, of *Clostridium acetobutylicum* are recommended for the tiered exemption.

VI. REFERENCES

7 CFR 330, et seq., as amended.

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